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Sensitive Detection of *C. parvum* using Near Infrared Emitting Ag_2S@Silica Core-Shell Nanospheres

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ABSTRACT

An optical immunosensor using anti-oocysts McAb Immobilized Near-Infrared (NIR) emitting Ag_2S@Silica core shell nanospheres for a detection of *C. parvum* in water were developed. Formation of the core Ag_2S and the shell SiO_2 over the core was confirmed by X-ray diffraction (XRD) and High Resolution Transmission Electron Microscope (HRTEM). The optical properties of the nanostructures are determined by UV-Vis spectroscopy and photoluminescence (PL) studies. The Core-Shell Ag_2S@Silica nanospheres exhibited an intense emission peak at 896 nm which falls on biological window. The TEM images confirms that the presence of uniform Core-Shell structure of Ag_2S having average size of 80 nm, and silica shell with thickness of 40-50 nm. The *C. parvum* antibody anti-oocysts McAb Immobilized Ag_2S@Silica nanoparticle was used as detector probe and biosensor exhibited excellent analytical performance toward the detection of *C. parvum* with detection limits of 10 oocysts /mL with minimal assay period.

Keywords: Biosensor, Core-Shell nanoparticle, photoluminescence, *C. parvum*. Ag_2S@Silica.

1. INTRODUCTION

Cryptosporidium Parvum is an obligate intracellular protozoan parasite infecting a wide range of vertebrate hosts such as humans, birds and cattles.[1-4] The quantification of *C. parvum*

oocysts in water samples can be done by ELISA protocol but the detection limit merely exists in 10,000 oocysts per gallon.[5] Conventionally, *Cryptosporidium parvum* can be detected by acid-fast staining,[6] immunofluorescent (IF) antibody staining,[7] flowcytometry,[8] and polymerase chain reaction (PCR).[9],[10] In immunofluorescent (IF) antibody staining has the non-specificity of the antibody due to the cross reaction with other species might be a problem and this method requires a large number of oocysts ranging from 50000-500000 oocysts /g in order to give a positive detection feed back.[11] The polymerase chain reaction (PCR) is able to detect oocysts number ranging from 100-1000 oocysts /g,[4] However, this process is time consuming and the chemicals needed are relatively expensive compared to other methods.

Though there are several advantages of organic dye labeled optical biosensor known, for instance, photostable, sensitive and water solublility, [12-14] the emission of organic dyes is susceptible to quenching through Fourier resonance energy transfer (FRET). In addition to FRET, traditional fluorescence based sensors requires long time of assay, sophisticated instrument, toxic chemicals of high cost that limits its use as tool for routine screening of pathogens in various biological samples. In order to overcome such limitations of fluorescence biosensors, quantum dots are recently employed as alternative materials to dye labeled sensors. Nanomaterials have great potential as attractive sensor materials for sensitive and quick detection because of their high surface-area-to-volume ratio and efficient interaction with analytes. It was reported that detection sensitivities of nanoparticle based biosensor are superior to those of conventional sensors.[15,16] Hence in the present work, we have synthesized antibody labeled NIR emitting Core-Shell Ag_2S@Silica nanospheres so as to use as detector probe, which offers the following advantages: In First, The silica shell can act as protective layer to Ag_2S core and the silica matrix is optically transparent that will allow excitation and emission light to pass through

the silica framework. Secondly, NIR emitting QD is expected to give more sensitive results because of the fact that biomolecules are highly transparent to NIR that avoids fluorescence intensity loss due to scattering of radiation by biomolecules.[17] The biological imaging of NIR emitting Quantum dots are studied in the literature[18, 19] However, The advantages of Core-Shell type nanospheres are not well exploited in immunofluorescent sensors so far. In this work, The optical features of NIR emitting Core-Shell Ag_2S@Silica Nanospheres has been exploited to fabricate optical immunosensor and studied the limiting sensitivity by taking *C. parvum* as a model pathogen.

2. EXPERIMENTAL

2.1 Materials and Methods

Parasitic *Cryptosporidium parvum* at a concentration of 1×10^5 oocysts /mL and anti-oocysts monoclonal antibody of 1/20–1/ 2000 unit were purchased from BTF Microbiology, Australia and Virostat, USA. Quartz glass, Cetyltrimethylammonium bromide (CTAB, 99.0%), Formaldehyde solution (37.0 wt%), Ammonium nitrate (99.0%), and Sodium sulfide (98.0%), GPTMS (3-Glycidoxypropylmethyldiethoxysilane), TEOS (Tetraethyl orthosilicate), BSA (Bovine Serum Albumin), PBS (phosphate buffer saline), absolute anhydrous ethanol (99.7%), Silver nitrate (99.8%) and Sodium hydroxide (96.0%) were purchased from Sigma-Aldrich and SRL, India. All chemicals were used without additional purification. Deionized water was used for all experiments. The optical properties were measured with an UV Spectrophotometer (Shimadzu UV-1800) and PerkinElmer LS 55 Fluorescence spectrometer. The morphology of Ag_2S@Silica nanospheres was investigated using a Technai10-philphs transmission electron microscope at 100 kV. Regular TEM specimens were made by evaporating one drop of Ag_2S@Silica nanospheres solution on carbon-coated copper grids.

2.2 Synthesis of Ag_2S@Silica Nanospheres.

In our experiment the Ag_2S@Silica nanospheres were synthesized by the simple one-pot process combined with sulfuration.[20] In brief, 0.10 g of CTAB was dissolved in the solution containing 96 mL of water and 0.7 mL of 0.5M NaOH and stirred at 80 °C for 30 min, and then 0.4 mL of 1.0M formaldehyde aqueous solution and 1.0 mL of 0.15M silver nitrate aqueous solution were added. To the resulting mixture 0.3 g of TEOS was added with stirring and a yellow precipitate was formed within several minutes. After stirring for 30 min, 1.0 mL of 0.1M sodium sulfide solution was added. The yellow precipitate turned black immediately. The products were filtered after further stirring for 2 h, washed by ethanol and water, and then dried at 50 °C in vacuum. The final product could be easily dispersed in ethanol and water.

2.3 Preparation of Anti-oocysts McAb Immobilized Ag_2S@Silica Nanospheres

For covalent conjugation of McAb with Ag_2S@Silica nanospheres, the surfaces of the prepared Ag_2S@Silica nanospheres were functionalized with 3-(Glycidoxypropyl) methyldiethoxy silane.[21] In brief, 5 mL of Ag_2S@Silica nanospheres suspension was stirred with 0.5 mL of 1% GPTMS for 6 more than hours, and kept at 4 °C for further use as stock. The GPTMS functionalized Ag_2S@Silica nanospheres were collected by centrifugation, and rinsed thoroughly with ethanol to remove any physically adsorbed GPTMS. One milliliter of GPTMS functionalized Ag_2S@Silica nanospheres from the stock solution was mixed with 100 μL of primary anti-oocysts McAb (10μg mL^{-1}) and stirred for an hour at room temperature; the unbound anti-oocysts McAbs were then removed by centrifugation and successive washing with PBS and finally the anti-oocysts McAb Immobilized Ag_2S@Silica nanospheres were dispersed in 1 mL of PBS.

2.4 Fabrication of Quartz glass plates for the detection of *C. parvum*

The Quartz glass plates (2 × 0.5 cm) were cleaned thoroughly in an ultra-sonication process using, acetone followed by water. The SAM (self- assembled monolayer) of GPTMS on the pre-cleaned Quartz glass plates was prepared as per the reported procedure.[3] The cleaned glass plates having dimensions of 2cm x 0.5cm were immersed in a solution of 1: 1: 5 (v/v) H_2O_2:NH_4OH:H_2O for 30 min at 80 °C for hydrolysis, and rinsed thoroughly with de-ionized water, followed by drying; the plates were then immersed in 1% of (v/v) 3-Glycidoxypropylmethyldiethoxysilane (GPTMS) for 20 hours. Subsequently, the plates were dried, and then immersed in 200 µL (1 mg/mL) of anti-oocysts McAb for an hour. The plates were then blocked with 3% BSA in phosphate buffer saline for another one hour at room temperature.

2.5 Optical Detection of *C. parvum* by Anti-oocysts McAb Immobilized Core-Shell Ag_2S@Silica Nanospheres.

The fabricated glass plates were then incubated with 200 µL of the target *C. parvum* antigens at room temperature for 30 minutes, and washed with a PBST buffer. After being dried, the glass plates were treated with 200 µL of anti-oocysts McAb Immobilized Core-Shell Ag_2S@Silica nanospheres for 30 minutes, and washed with PBST to remove the nonspecific binding on the glass plate surface. The modified quartz glass plates were analyzed with a Fluorescence spectrometer.

3. RESULTS AND DISCUSSION

3.1 Characterization of the anti-oocysts McAb Immobilized Core-Shell Ag_2S@Silica Nanospheres

The Core-Shell Ag_2S@Silica nanospheres as prepared were analyzed using Uv spectroscopy, fluorescence spectrometer, High-resolution transmission electron microscopy

(HRTEM), and x-ray diffractogram. The UV/Vis absorption spectra of Ag_2S@Silica Colloid solution and anti-oocysts McAb Immobilized Ag_2S@Silica are presented in Fig.1. Absorption peaks observed at 242 nm is assigned to sulfide functional group. A less intense shallower band at 350 nm is attributed to silver[22] which indicate the formation of monodispersed Ag_2S@Silica nanospheres. Following antibody conjugation, the absorption shifted to the red indicating the conjugation of the antibody.

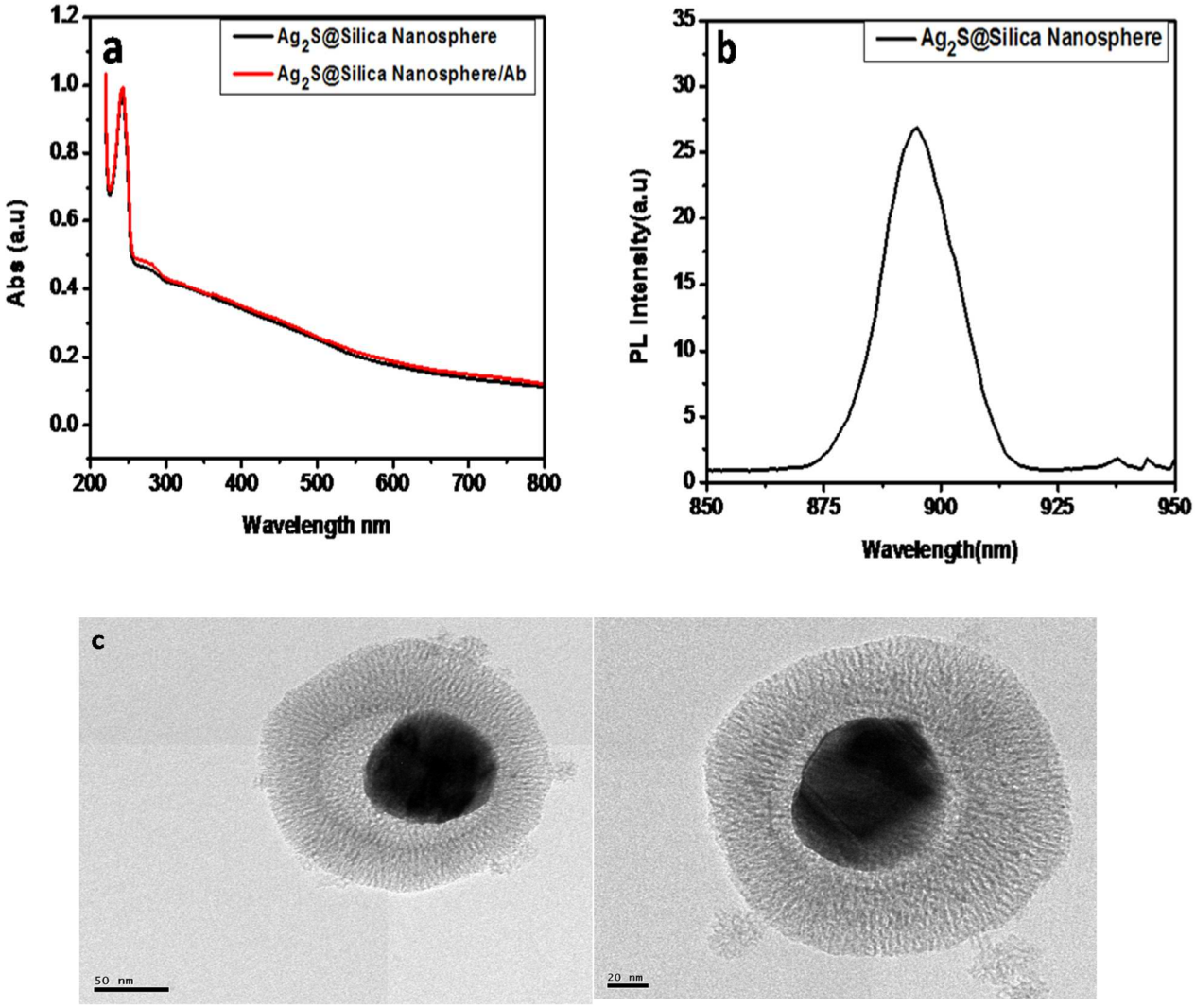


Fig. 1 (a) UV spectral analysis of Ag_2S@Silica and anti-oocysts McAb Immobilized Ag_2S@Silica Core shell Nanospheres. (b) PL spectral analysis of Ag_2S@Silica Core shell

Nanospheres. (C) Transmission Electron Microscopic image of Ag_2S@Silica Core shell Nanospheres

The photoluminescence studies (Fig.1b) shows that the core-shell nanospheres exhibits an intense emission at NIR region having a emission maximum at 896nm, exactly falls at the biological window (700-1100nm), which is anticipated to enhance the sensitivity of detection due to the fact that radiation within the biological window have maximum penetration in tissue and interference of tissue auto fluorescence is minimal. The TEM images of Ag_2S@Silica Core shell Nanospheres were presented in Fig. 1c. A typical Core-Shell structure was clearly observed, with a single silver sulfide nanoparticle core and uniform silica shell with an average diameter of 120 nm. Further, it is observed that the Ag_2S core has the average size of 80 nm and silica shell thickness ranges from 40-50nm.The X-ray diffractogram of Ag_2S, $Ag_2S@SiO_2$ are presented in the Fig 2. The peaks at 31.4° (d = 2.80 Å), 34.3° (d = 2.60 Å), and 36.8° 2θ (d = 2.40 Å) are in good agreement with the characteristic peaks of monoclinic Ag_2S (JCPDS Card File 14-0072), corresponding to the (−112), (−121), and (121) planes. Further, the presence of silica shell was also confirmed from the existence of broad peak at 2θ = 22° in Fig. 2c which is due to the reflection from SiO_2 surrounding around Ag_2S core. The XRD results are in consistent with the TEM micrograph illustrated in Fig.1b.

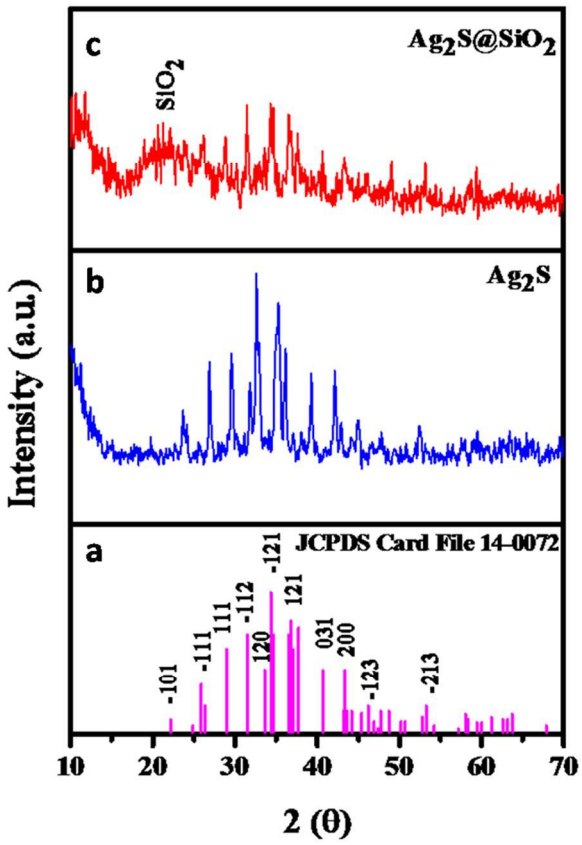
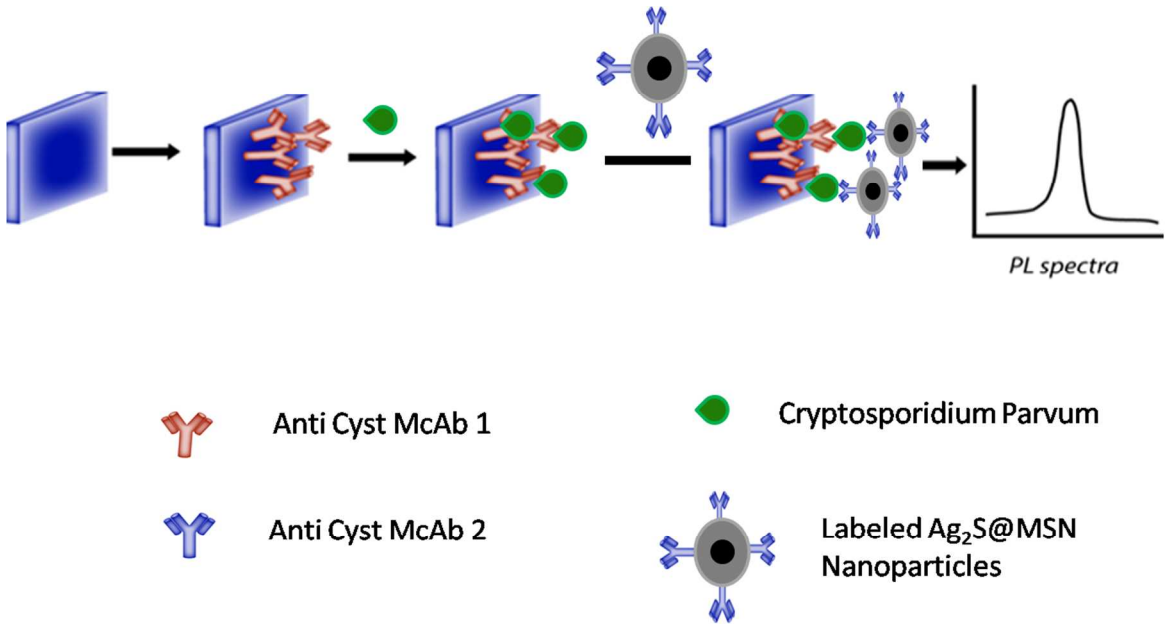


Fig. 2 XRD pattern of the (a) monoclinic Ag_2S phase (JCPDS Card File 14-0072). (b) Ag_2S nanoparticles (C) Ag_2S@Silica Core shell Nanospheres.

3.2 Principle of anti-oocysts McAb Immobilized NIR emitting Core-Shell Ag_2S@Silica nanospheres based optical immunosensor

The principle of the anti-oocysts McAb Immobilized NIR emitting Core-Shell Ag_2S@Silica nanospheres based optical immuno sensor is based on the sandwich form of the antibody immuno assay. As shown in scheme 1, anti-oocysts McAb was immobilized on the 3-(Glycidoxypropyl) methyldiethoxy silane functionalized quartz glass plate and subsequently incubated with the target *C. parvum*. The glass plates were then treated with anti-oocysts McAb Immobilized Core-Shell Ag_2S@Silica nanospheres and it was used to measure photoluminescence (PL) spectra to detect the presence of target pathogen.



Scheme 1. Principle of anti-oocysts McAb Immobilized NIR Emitting Core-Shell Ag_2S@Silica Nanospheres Based Optical Immuno Sensor

3.3 Optical characterization of the modified quartz glass for *C. parvum* detection

The consecutive immobilization of GPTMS, primary anti-oocysts monoclonal antibody, oocysts and anti-oocysts McAb Immobilized Core-Shell nanoparticles on the quartz glass were characterized by PL spectroscopy. As shown in Fig. 3 the PL peak was not observed in GPTMS, the anti-oocysts antibody, and the oocysts treated glass slide. The PL spectrum appeared at 896 nm on the anti-oocysts McAb Immobilized Core-Shell nanoparticles treated glass plate, which indicates the presence of the target *C. parvum* on the modified glass plate, but it was not in the absence of the *C. parvum,*. The resulting appearance of an intense PL is attributed to a specific detection of *C. parvum* on the antibody functionalized glass surface, and followed by treatment with the anti-oocysts McAb Immobilized core shell nanoparticles. Consequently, the obtained PL spectrum confirmed that the different species were sequentially immobilized on the GPTMS functionalized quartz glass.

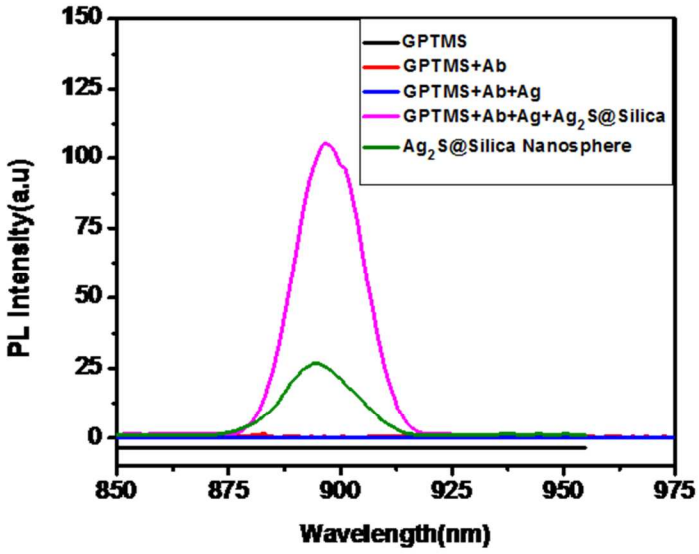


Fig. 3 PL spectral analysis of modified quartz glass to develop NIR emitting Core-Shell Nanoparticles based immunosensor

3.4 Efficiency of the NIR emitting Core-Shell Ag_2S@Silica Nanospheres based optical immunosensor for the detection of *C. parvum*

The developed NIR emitting Core-Shell silica nanomaterial based optical immuno assay was tested for sensitivity and selectivity for the detection of model analyte *C. parvum*. A serial numbers for instance 0 to 100 oocyst/mL were used and analyzed to quantify the assay efficiency. The optical response for the analysis of oocysts with serial numbers is shown in the Fig. 4a and 4b. It was found that the photoluminicent absorbance peak was increased as expected with increased the number of oocyst, also shows linear response from 10 to 100 oocyst/mL whereas below 10 oocyst/mL (5 oocyst/mL) little deviation in linearity was found. This may be due to the limited count of the target C. parvum. The optimized linearity came to exist only after 10 oocyst/mL and the limit of detection 10 oocysts /mL was noted. The sequential treatment of quartz glass, as shown in scheme 1, except oocyst did not show any photoemission which reveals that the appearance of a emission peak in Figure 3 is not due to the non-specific or physical

adherence of core shell nanospheres on quartz glass. The reproducibility of the developed optical immuno sensor was assessed by a series of 5 immuno assays under optimized conditions, to detect 100 & 50 oocyst/mL. The coefficient variations of the assay are 4.1% and 2.7% respectively, which suggested that the assay is reproducible in optimized conditions. Further, the developed NIR emitting Core-Shell silica nanomaterial based optical immuno assay was validated by studying the specific binding of anti-oocysts McAb immobilized Ag_2S@Silica Nanospheres with other pathogens such as *Giardia, Vibrio Cholerae, Shigella, E.coli* and *Salmonella,* and it was found that the photoemission is not observed for none of the pathogens except *C. parvum* (Figure 5).

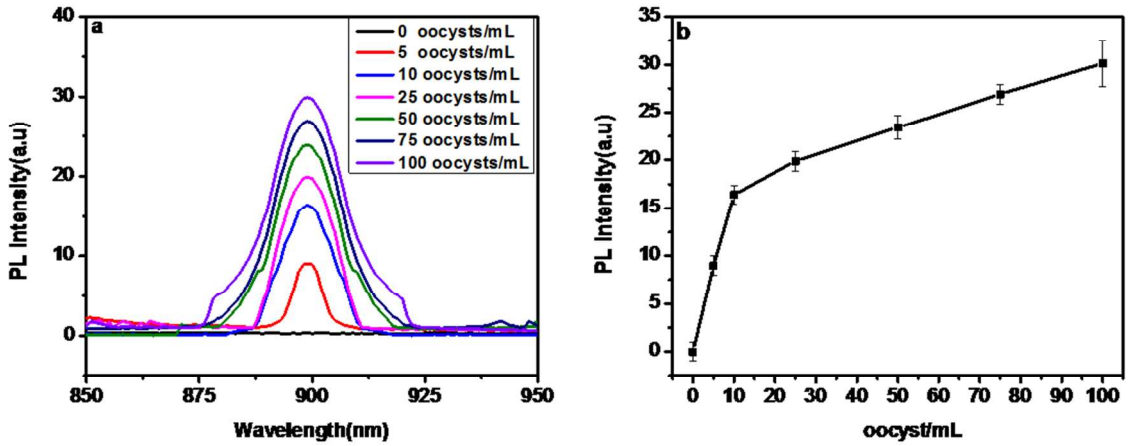


Fig. 4 PL analysis of anti-oocysts McAb Immobilized NIR emitting Core-Shell Ag_2S@Silica Nanospheres based immunosensors for *C. parvum* detection. (a) Various concentrations of *C. parvum* used from top to bottom of 0 to 100 oocysts /mL of *C. parvum* and (b) Standard graph of PL intensity versus the concentration of *C. parvum* (oocysts /mL) using NIR emitting Core-Shell Nanoparticles.

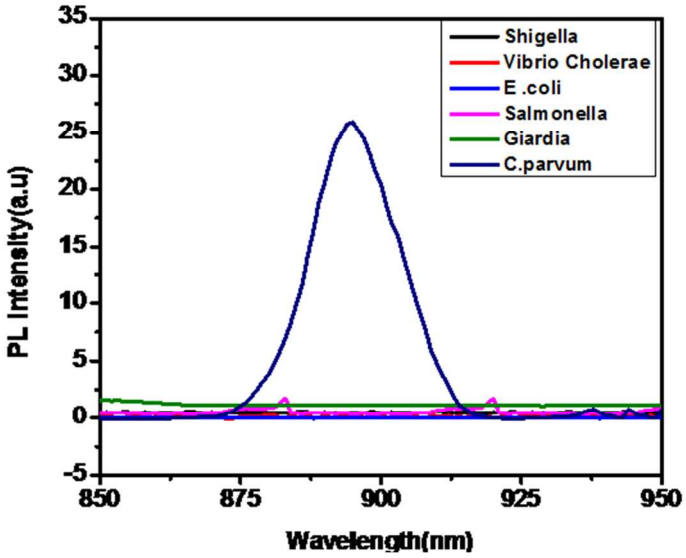


Fig. 5 Validation of specificity of the developed anti-oocysts McAb Immobilized NIR emitting Core-Shell Ag_2S@Silica Nanospheres based immunosensor assay for detection of *C. parvum* i) *Shigella,* ii) *Vibrio Cholerae,* iii) *E coli*, iv) *Salmonella,* v) *Giardia*, and vi) *C. parvum*

4. CONCLUSION

We have utilized the optical features of NIR emitting Core-Shell Ag_2S@Silica Nanospheres to fabricate an optical immunosensor and studied the limiting sensitivity by taking *C. parvum* as a model pathogen. The study reveals that the developed optical immunosensor can detect *C. parvum* as low as 10 oocysts /mL with minimal assay period. We conclude that the anti-oocysts McAb Immobilized NIR emitting Core-Shell Ag_2S@Silica Nanospheres can be employed for the optical immuno sensing of *C. parvum* in environmental samples.

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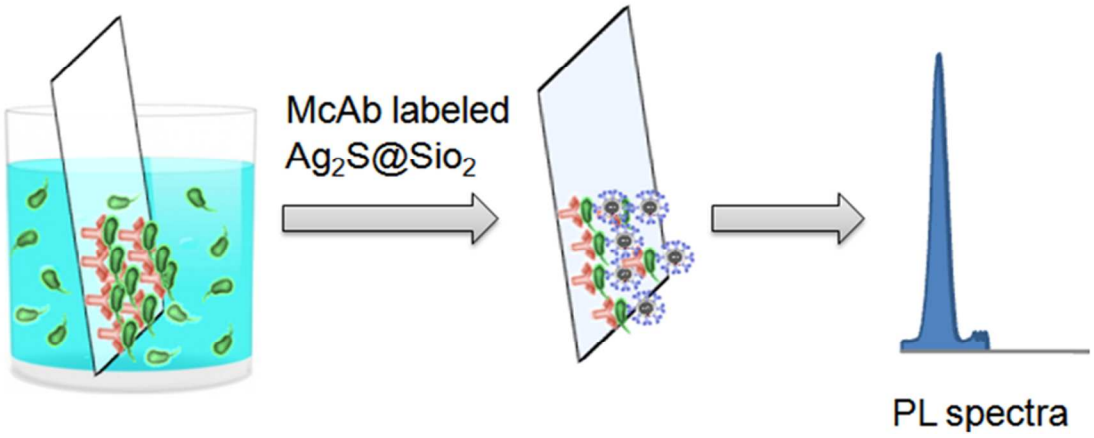

Optical detection of C.parvum in environmental samples was reported using anti-oocysts McAb immobilized NIR emitting Ag_2S@Silica core shell nanospheres as immunosensor.